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Original Article

The association of inhibitor of Growth 4 with its prognostic value in osteogenic sarcoma

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Abstract: Background: Osteosarcoma is one of the first two cause of cancer-related death in children and young adolescents. Inhibitor of Growth 4 (ING4) is a member of the ING tumor suppressor family and play an important role in many cellular processes. The purpose of this study was to explore the correlation between *ING4* expression and the prognosis of osteosarcoma patients. Methods: *ING4* mRNA and protein expressions in osteosarcoma and normal tissues were detected by quantitative real-time transcriptase polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC) method, respectively. Chi-square test was adopted to estimated the relationship of *ING4* expression and clinical parameters of osteosarcoma patients. Besides, the overall survival of osteosarcoma patients was evaluated by Kaplan-Meier method. The potential of *ING4* as a prognostic marker gene was addressed by Cox regression analysis. Results: Down-regulated expression of *ING4* mRNA and protein were observed in osteosarcoma tissues. *ING4* expression was significantly associated with metastasis ($P = 0.030$) and recurrence ($P = 0.008$), but not other clinical features ($P > 0.05$). Cox regression analysis indicated that *ING4* can be used as an independent prognotic biomarker for osteosarcoma, in univariate and multivariate analysis ($P = 0.004$, HR = 3.945, 95 % CI = 1.565-9.940; $P = 0.001$, HR = 4.213, 95 % CI = 1.747-10.161). Conclusion: Taken together, *ING4* was down-regulated in osteosarcoma tissues. *ING4* can act as an independent prognostic factor for osteosarcoma.

Keywords: *ING4*, prognosis, osteosarcoma

Introduction

Osteosarcoma, the most common primary bone malignancy [1-4], is one of the first two cause of cancer-related death in children and young adolescents [5]. Osteosarcoma is a complex disease. The development of osteosarcoma is a multistep and multifactorial process, implicating many factors [6-8]. Since it was discovered, the therapy method of osteosarcoma was continuously explored. However, the survival rate of the osteosarcoma patients is quite poor. Distant metastases to the lung and the local recurrence are the most common phenomenon after the surgery for osteosarcoma. In order to improve the prognosis of osteosarcoma, many treatment methods were carried out. Recently, molecular target therapy for tumors has been employed in the clinical treatment [9], which will promote and broaden the underlying application prospects of tumor target therapy in future.

Inhibitor of Growth 4 (ING4) is a member of ING family, which comprises 5 members, including ING1, ING2, ING3, ING4 and ING5. ING4 encode by *ING4* gene, it is localized at chromosome 12p12-13 region [10] and encodes a 249-amino acid protein containing a highly conserved C-terminal plant homeodomain finger motif (PHD) and 2 nuclear localization signals [11]. It is reported that ING4 play an important role in many cancer-related cellular processes including cell proliferation, apoptosis, migration, angiogenesis, contact inhibition, DNA damage response, and hypoxia [12-19]. Numerous studies have revealed the suppressive role of *ING4* in various cancers, including breast cancer, gastric carcinoma, colon cancer, non-small cell lung cancer, ovarian carcinoma, head and neck squamous cell carcinoma, melanoma, hepatocellular carcinoma [20-27]. Although it has been demonstrated that *ING4* gene significantly inhibits proliferation and invasion and

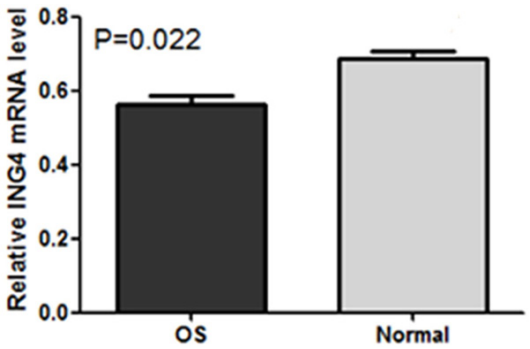

Figure 1. The relative *ING4* mRNA expression level in osteosarcoma and normal tissue.

promotes apoptosis of human osteosarcoma cell [28], there were few studies on the correlation between *ING4* gene and prognosis of osteosarcoma.

In the present study, we aimed to explore the potential role of *ING4* gene in the prognosis of osteosarcoma patients.

Materials and methods

Patients and tissue samples

89 osteosarcoma tissues and adjoining normal tissue samples were obtained from patients who were diagnosed as osteosarcoma in The First Affiliated Hospital of Chongqing Medical University from 2005 to 2009. All patients had not received chemotherapy or radiotherapy prior surgery. A 5-years' follow-up was conducted and the information was updated via a telephone or questionnaire. The death of the participants was ascertained by a report from the family and verified by the review of public records. The study was approved by the ethics committee of The First Affiliated Hospital of Chongqing Medical University. Written informed consent was obtained from all participants. The samples were quickly frozen in liquid nitrogen and stored at -80°C until use.

RNA preparation, and quantitative real-time transcriptase polymerase chain reaction (qRT-PCR)

Total RNAs were extracted from tumor tissue and paired normal tissue samples using Trizol (Takara, Dalian, China). First-strand cDNA synthesis was performed using the Superscript III kit (Life Technologies, USA). qRT-PCR was performed using a One Step SYBR® PrimeScript® RT-PCR Kit (Takara) following the manufacturer's instruction. Human *β-actin* was amplified as internal PCR control. Sequences of the primers were as follows: *ING4*, 5'-TCG TGC TCG TTC CAA AGG-3' and 5'-GGC AAT AGG TGG GTT CGT T-3' [29]; human *β-actin* forward 5'-TGA CGT GGA CAT CCG CAA AG-3' and reverse 5'-CTG GAA GGT GGA CAG CGA GG-3' [30]. The real-time PCR was performed with an Applied Biosystems 7900 (Applied Biosystems, USA). The expression level of *ING4* was evaluated by comparative cycle threshold (CT) method.

Immunohistochemistry (IHC) analysis

IHC method was utilized to detect the *ING4* protein expression in osteosarcoma samples. Immunohistochemistry procedure was performed as described previously [27]. Tumor and normal tissues were cut into 4 μm sections after formalin-fixed, paraffin-embedded blocks. Then, tissue sections were dewaxed at 55°C for 30 min and washed three times with xylene and rehydrated in graded alcohol, followed by two washes in distilled water. Antigen retrieval was performed by heating the samples at 95°C for 30 min in citrate buffer (10 mM, pH = 6). Endogenous peroxidase activity was blocked with 3 % hydrogen peroxide in methanol for 30 min. Blocking with universal blocking serum for 30 min was used to reduce background nonspecific staining. Sections were incubated overnight at 4°C with a goat anti-human polyclonal antibody against ING4 (Abcam; 1:200 dilution). Second antibody (rabbit anti-goat antibody; MaiXin Bio) was applied for 45 min at 37°C, and then incubated with DAB (Golden Bridge Int.) to visualize *ING4* expression. Formalin-fixed paraffin-embedded human brain tissues, which express ING4 in glial cells, were used as internal positive controls. Negative staining controls were obtained by omitting the primary antibody.

Intensity of cytoplasmic staining was scored as 1 to 4, by comparison to the positive internal controls. Percentage of ING4-positive stained cells was: 1 (0-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). This scoring system has been previously validated [31, 32]. Diffuse, moderate to strong cytoplasmic staining characterized ING4-positive cells (scores 3 and 4). ING4-negative cells were devoid of any cytoplasmic staining or contained faint, equivocal staining (scores 1 and 2).

Table 1. Expressions of *ING4* protein in osteosarcoma and normal tissues

Tissues	Number	*ING4* protein expression		X^2	*P* value
		Positive	Negative		
Normal	89	60 (67.42%)	29 (32.58%)	/	/
OS	89	47 (52.81%)	42 (47.19%)	3.960	0.047

Statistical analysis

All statistical analysis were carried out using the software of SPSS version 18.0 for Windows (SPSS Inc, IL, CA, USA). Differential expression of *ING4* between osteosarcoma tissues and paired normal tissues was evaluated by paired sample t test. Chi-square test was used to analyze the relationship between *ING4* protein expression and the clinicopathological characteristics. Kaplan-Meier analysis was used to estimated the overall survival rate of the osteosarcoma patients with different *ING4* expression, differences were calculated using the log rank test. Hazard ratios (HRs) with 95% confidence intervals (95% CI) for the time-to-event endpoint were estimated using the multivariate Cox regression analysis in a forward stepwise method to evaluate the effect of multiple independent prognostic factors on survival outcome. Differences were considered statistically significant when *P* value < 0.05.

Results

ING4 expression is decreased in osteosarcona

QRT-PCR was used for evaluating *ING4* mRNA level in 89 paired tumor and normal tissues. The relative expression of *ING4* mRNA was 0.653 ± 0.029 in osteosarcoma tissues and 0.731 ± 0.019 in the paired normal tissues. The difference was significant (**Figure 1**, *P* = 0.022). IHC assay was used for evaluating *ING4* protein expression, 52.81 % osteosarcoma samples were ING4-positive (47 of 89), that was significantly less than normal samples (67.42%, 60 of 89, *P* = 0.047, **Table 1**).

Correlation between ING4 expression and clinicopathologic features of osteosarcoma

According to the results of IHC, the osteosarcoma cells were divided into two groups: ING4-positive group and ING4-negative group. The IHC data from 89 osteosarcoma specimens were analyzed for the correlation of ING4 levels with clinicopathologic features. The results were summarized in **Table 2**. Most of the non-metastasis osteosarcoma tissues performed as ING4-positive tissues (62.75%), meanwhile only 39.47% metastasis osteosarcoma tissues were performed as ING4-positive (*P* = 0.030). About 38.64% local recurrence osteosarcoma tissues were ING4-positive tissues, 66.67% non-recurrence osteosarcoma tissues were performed as ING4-positive tissues (*P* = 0.008). No significant association was found between *ING4* protein expression levels and the following clinical features in the osteosarcoma tissues, such as age, gender, WHO grade and tumor site (all, *P* > 0.05).

Association of ING4 expression with prognosis of osteosarcoma patients

Correlation between *ING4* protein expression and survival time of the patients with osteosarcoma was evaluated by Kaplan-Meier survival analysis. Mean follow-up period of all patients with osteosarcoma in this study was 38.18 months (range from 3 to 60 months). During the follow-up period, 20 of 42 patients with ING4 negative expression (47.62%) had died, whereas 10 of 47 ING4 positive expression patients (21.28%) had died. The result of Kaplan-Meier survival analysis indicated that patients with ING4-negative expression had worse overall survival than those with ING4-postive expression, log-rank test suggested that the difference was significant between ING4-positive and ING4-negative group (*P* < 0.001) (**Figure 2**). Multivariate Cox analysis indicated that *ING4* was an independent prognostic factor (**Table 3**, *P* = 0.001, HR = 4.213, 95% CI = 1.747-10.161).

Discussion

Osteosarcoma is a most common malignancy in children and young adolescents. This tumor has a poor prognosis, such as the rapid growth, lower cure rate and easy relapse. Recent years, many investigators devote themselves to looking for an effective therapy method for osteosarcoma, so as to enhance the survival rate of patients. A novel prognosis factor will contribute to solving this issue. *ING4* as a novel mem-

Table 2. Correlation between *ING4* expression and clinicopathologic features of osteosarcoma

Characteristics	Numbe of case	*ING4* expression				*P* value
		Positive (n = 47)	%	Negative (n = 42)	%	
Age						0.708
< 20	49	25	51.02	24	48.98	
≥ 20	40	22	55.00	18	45.00	
Gender						0.822
Male	54	28	51.85	26	48.15	
Female	35	19	54.29	16	45.71	
Tumor site						0.986
Femur	27	15	55.56	12	44.44	
Tibia	23	12	52.17	11	47.83	
Humerus	21	11	52.38	10	47.62	
Other	18	9	50.00	9	50.00	
WHO grade						0.074
Low (I, II)	45	28	62.22	17	37.78	
High (III, IV)	44	19	43.18	25	56.82	
Metastasis						0.030
Absent	51	32	62.75	19	37.25	
Present	38	15	39.47	23	60.23	
Recurrence						0.008
Absent	45	30	66.67	15	33.33	
Present	44	17	38.64	27	61.36	

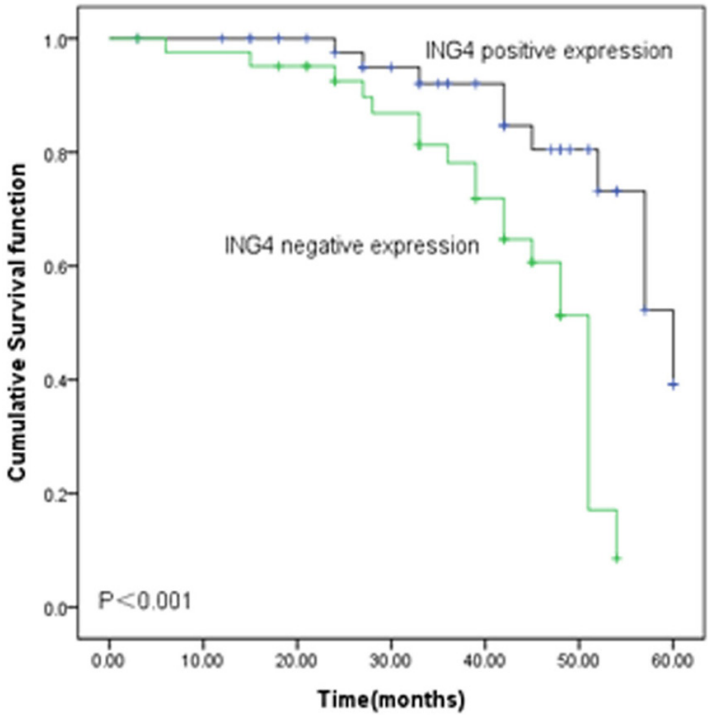

Figure 2. Kaplan-Meier analysis for patients with osteosarcoma based on *ING4* expression.

ber of ING family has potential tumor-suppressive effects. *ING4* is frequently mutated and its expression is decreased in various types of human cancers [33]. In previous study, the role of overexpression of *ING4* in osteosarcoma cell and *ING4* expression level in osteosarcoma tissues were studied by Li M et al. [28] However, its prognostic value in osteosarmonain patients was still unclear.

We carried out this study to investigate the correlation between *ING4* protein expression level and prognosis of osteosarcoma. QRT-PCR and IHC analysis results indicated that *ING4* expression was down-regulated in osteosarmona tissue than that in normal tissue. This findings are consistent with these earlier publications [28]. So *ING4* is an tumor suppressor gene. Then, we thought that *ING4* might has a potential role in the prognosis of osteosarcoma, and carried out a further study to solve this issue.

In the following work, we explored the correlation of the *ING4* protein expression and clinicopathologic characteristics of osteosarcoma patients. The results demonstrated that *ING4* protein expression level and distant metastasis of the tumor had an inverse correlation. Meanwhile, *ING4* protein expression were significantly lower in osteosarcoma patients with recurrence compared to patients without recurrence. This result was consistent with the previous study in in head and neck squamous cell carcinomas, brain tumour, ovarian carcinoma and lung cancer [10, 15, 24, 29]. The results provided a further evidence to testify that *ING4* can be used as a marker to determine the progression of osteosarcoma.

In the present study, correlation between *ING4* expression and

Table 3. Univariate and multivariate analysis of prognostic factors in osteosarcoma

Characteristics	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*	HR	95% CI	*P*
ING4 negative expression	3.945	1.565-9.940	0.004	4.213	1.747-10.161	0.001
Age	1.137	0.506-2.554	0.756	-	-	0.408
Gender	1.268	0.594-2.709	0.539	-	-	0.488
Tumor site	1.432	0.596-3.441	0.422	-	-	0.604
WHO grade	1.251	0.555-2.821	0.589	-	-	0.431
Metastasis	2.283	0.999-5.216	0.050	-	-	0.088
Recurrence	1.998	0.875-4.559	0.100	-	-	0.252

overall survival of the patients with osteosarcoma was evaluated by Kaplan-Meier survival analysis. Patients with ING4 positive expression had longer survival time and higher survival rate than ING4 negative expression patients. The results showed that *ING4* could be a prognostic factor, so we did further Cox regression analysis. The result indicated that *ING4* was an independent prognostic factors.

In conclusion, the *ING4* can be an independent prognostic marker to predict the unfavorable prognosis of osteosarcoma patients. Although we get a specific result, but our study sample size is too small, so the obtained results may be not accurate. Therefore, further studies with a large sample size are required.

Disclosure of conflict of interest

None.

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